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Project Title: Genetic and morphological analysis of species limits in *Cicurina* spiders (Araneae, Dictynidae) from southern Travis and northern Hays counties (TX), with emphasis on *Cicurina cueva* Gertsch and relatives

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Technical Report Schedule:
Preliminary report due 1 March, 2005
Final report due 1 May, 2005

Information in the subject report was provided by contractors to the Service, but at this point does not represent a Service position

Summary of Project Results:

We have combined genetic and morphological data to understand species limits in **cave-limited** *Cicurina* spider species (Araneae: Dictynidae) from caves in central Texas. Particular emphasis is placed on *Cicurina cueva* and close morphological relatives (*C. reyesi* and *C. bandida*) found in southern Travis and northern Hays counties. The project included the collection of specimens from the field, illustration and qualitative comparison of morphological variation, and molecular phylogenetic analysis of mitochondrial DNA sequence data. Our primary findings can be briefly summarized as follows:

When analyzed and compared to a comprehensive sample of *Cicurina* taxa from the region (Travis, Hays & Williamson counties, TX), sampled populations of *C. cueva* form a monophyletic group (clade) on a mitochondrial phylogeny. Populations representing both *C. bandida* and *C. reyesi* are deeply embedded within this *C. cueva* genetic clade. This observation, in addition to consideration of female genital morphology, suggests that these three named taxa represent **variants of a single species**.

This single species is **not** genetically homogeneous (as might be predicted if there were high levels of gene flow across caves), but instead shows geographic-based genetic structuring. This finding makes biological sense, as we would expect geographically-adjacent cave populations to share more genetic similarity than caves that are distant in space. The genetic structuring observed is a natural consequence of the fragmented nature of cave habitats, and the unique habitat limitations of these spiders (these spiders are cave-restricted and totally eyeless).

Despite an overall pattern of geographic-based genetic structuring, there is some evidence for gene flow between caves and cave systems. Furthermore, there is **no** predictable pattern of morphological variation associated with geography. A similar genital morphology, with slight variations, is shared across the entire distribution of this species.

We do not formally propose synonymy in this report, as such a formal taxonomic decision must be based on publication in a scientific journal. Instead, in this report we informally refer to the morphologically variable and genetically divergent populations within this single species as the “*C. cueva* complex”. Members of this complex are found in over 20 caves from a geographically-confined region. Despite the morphological and genetic variation observed, this single species is clearly distinct and easily distinguished from other *Cicurina* species in the region.

Final Report (initial draft completed 4 May, 2005)

Background & Research Approach:

Cicurina cueva Gertsch 1992 (Araneae: Dictynidae) is a rare, troglomorphic spider described from Travis County, Texas. Before this study, this spider species was known only from two, geographically-separated, cave locations (Cave X and Flint Ridge Cave, see **Fig. 3**), from a total of three adult female and several immature specimens (these are the published records from Gertsch 1992). The apparent rarity of this spider species in a rapidly-developing region has prompted conservation concern. Effective conservation activities require that we understand species limits and the full geographic distribution for all *Cicurina* spiders of the region. Both pieces of taxonomic information are fundamental for making informed conservation decisions and setting conservation priorities.

This study attempts to clarify the species limits and geographic distribution of cave-dwelling *Cicurina* of southern Travis county and adjacent areas, via combined analysis of molecular, morphological and geographic evidence. We have conducted similar analyses for endangered *Cicurina* from Bexar County, Texas, and have published these results (Paquin & Hedin, 2004). The research strategy summarized in this report follows that of Paquin & Hedin (2004) rather closely.

Data & Methods:

Collections - Eyeless *Cicurina* specimens were collected from about 70 caves in Travis, Williamson and Hays counties, Texas. Fieldwork was conducted from 4-14 January 2005 and 2-16 March 2005. A total of 275 *Cicurina* specimens were collected, including 4 adult males and 44 adult females. Females are the more morphologically-informative sex in this genus (Gertsch 1992), and this number of females **greatly** increases the number of adult specimens available for study. Collected spiders were preserved in 100% EtoH, stored cold while in the field, and are currently housed in a -20C freezer in the Hedin lab at SDSU. All collections were conducted (or supervised) by Dr. Pierre Paquin, except collections from Lamm Cave, Tooth Cave and Shell Spur Cave. These spiders were collected by Jean Krejca (Lamm and Tooth caves) and James Reddell (Shell Spur Cave), and generously donated to this study. Because these caves do not house the species of primary interest in this report, data from these specimens are not reported here.

Morphological Analyses - We dissected and illustrated female genitalia for all adult female specimens from our new collections. In addition, we have borrowed type specimens for the most relevant taxa (*C. cueva* and *C. bandida*), and have dissected and

re-illustrated genitalia for these specimens.

Molecular Data & Analysis - We have subsampled our total specimen sample so as to maximize the number of caves sampled, rather than the number of individuals sampled per cave. When available, two individuals were selected per cave for DNA analysis, except for key localities where all available individuals were included (Flint Ridge, Cave X, Cave Y, Blowing Sink, Ireland's Cave). DNA extraction, PCR amplification of the mitochondrial CO1 gene, and PEG purification of these PCR products were completed using general procedures detailed in Paquin & Hedin (2004). DNA sequence data were edited and aligned, then analyzed using modern phylogenetic methods (see Paquin & Hedin, 2004). Analytical details are available upon request from the authors.

Results:

Below we present our primary findings as they relate to the main objective of this research, which is to understand the species limits and distribution of *C. cueva* and close relatives.

1) When analyzed together with other *Cicurina* taxa from the region, individuals from caves representing *C. cueva* form a well-supported monophyletic grouping (a clade) on a mitochondrial gene tree (**Fig. 1**). More thorough Bayesian phylogenetic analyses of a relevant subset of DNA data (i.e., excluding obviously distantly-related taxa) shows that individuals from caves representing known populations of *C. cueva* (Cave X and Flint Ridge Cave), *C. bandida* (Ireland's Cave), and *C. reyesi* (Airman's Cave) are embedded in this genetic clade (**Fig. 2**). This "*C. cueva* complex" clade has a clearly defined geographic distribution in southern Travis and northern Hays counties (**Fig. 3**). Compared to our earlier studies (Paquin & Hedin, 2004), the overall amount of genetic divergence within the "*C. cueva* complex" is similar to that observed in other *species* of eyeless *Cicurina* (**Table 1**), even with our larger sample size from the "*C. cueva* complex".

2) The *C. cueva* complex is not genetically homogeneous. If this group of populations was genetically homogeneous (i.e., no association between genetic variation and geographic location), this would suggest high levels of gene flow between caves in this system. This is not the case. Instead, the data reveal six well-supported mtDNA subclades (**Fig. 2**) that are geographically cohesive, meaning that the caves represented in the subclades are geographically adjacent in space (**Fig. 3**). This implies that historical and/or current levels of gene flow are constrained by geography, such that spider populations from caves within a geographic subunit are more genetically similar than populations from caves between subunits. This genetic structuring is expected in cave-restricted *Cicurina* populations (see Paquin & Hedin 2004), where the discontinuous

nature of the habitat places obvious restrictions on gene flow. In such systems, we expect geographically-adjacent cave populations to share more genetic similarity than caves that are distant in space. Our results are consistent with this expectation.

3) Despite this general pattern of geographic-based genetic structuring, in a few cases mitochondrial lineages (either the same or closely-related DNA sequences) are spread across space (**Fig. 2**). This is certainly the case within mtDNA subclades (e.g., some individuals from Cave X carry sequences that are more closely related to individuals in Cave Y than other individuals in Cave X - see **Fig. 2**, blue clade). Phylogenetic analysis also reveals that some caves house individuals from multiple mitochondrial lineages (**Fig. 2**, red & blue dots). Although other explanations are possible (e.g., incomplete lineage sorting), these points of evidence are most consistent with historical and/or on-going gene flow across caves in this system. Because our genetic sampling strategy was not designed to estimate such parameters (i.e., the number of individuals sampled per cave is low), we have not attempted to estimate the absolute magnitude of gene flow in this system.

4) Consideration of female genital morphology suggests that members of the *C. cueva* complex are clearly different from other taxa in the region (**Fig. 4**). These comparisons also indicate that *C. cueva*, *C. bandida*, and *C. reyesi* share very similar genital morphologies (see type specimens re-illustrated in **Fig. 5**). This is not only consistent with the close phylogenetic relationship indicated by mtDNA, but indicates to us that these named taxa actually represent a **single species** (see synonymy discussion below). Finally, consideration of morphology reveals that there is no predictable pattern of morphological variation associated with either geography or recovered genetic clades within the complex (**Figs. 6 - 9**). Instead, it appears that a similar genital morphology, with slight variations, is shared across the entire distribution of this single species. This is further evidence for shared history and historical/on-going gene flow in this system.

Taxonomic considerations:

When Gertsch (1992) described the three species *C. cueva*, *C. bandida*, and *C. reyesi*, his species hypothesis was based on a **very small number** of specimens from very few geographic locations. This included one adult female from Airman's Cave (= *C. reyesi*), two adult females from both Bandit Cave and Ireland's Cave (= *C. bandida*), and two adult females from both Cave X and Flint Ridge Cave (= *C. cueva*). Thus, the existing taxonomy of this group of species is based on **nine total** specimens. With this limited number of specimens one might imagine that it would be difficult to assess and interpret geographic variation in morphology. In fact, Gertsch (1992) referred to some specimens from both Ireland's and Flint Ridge Cave as "aberrant", because these specimens did not

conform to his “view” of the species. We also point out that the overlapping geographic distributions of his purported taxa (*C. cueva* in Cave X and Flint Ridge Cave, *C. bandida* in Bandit Cave and Ireland’s Cave; see **Fig. 3**) make little geographic sense.

Our new collections and new data allow us to assess this taxonomic situation in a more rigorous manner. These collections have more than doubled the number of adult female specimens for the taxa and region of interest. Consideration of these new specimens (including material from the type locality of *C. reyesi* and *C. cueva*), plus examination of the **type specimens** of *C. cueva* and *C. bandida*, suggest to us that female genital morphology cannot be used to separate three distinct taxa in this region. Instead, we interpret the morphological variation seen as evidence for geographic variation within a **single species**. This single species interpretation is consistent with phylogenetic analyses of the mtDNA data.

Conclusions & Significance:

Our systematic research on this group of rare spiders is of fundamental importance to regional conservation efforts. Using a combination of DNA, geographic and morphological evidence, we have constructed a data-rich hypothesis that allows us to understand species limits in these cave-dwelling *Cicurina*. Our findings suggest that a **single species**, informally referred to as the “*C. cueva* complex”, is restricted to a small region in southern Travis and northern Hays counties, is found in over 20 caves in this region, and is both morphologically and genetically variable. Despite this variation, this species is both morphologically and genetically distinct from other taxa in the region, and can be easily distinguished from such taxa. **We suggest that conservation activities concerning cave populations in this confined geographic region be based on this single species hypothesis.**

Future Directions:

A formal taxonomic decision such as species synonymy must involve publication in a scientific journal. As such, we do not propose a formal synonymy in this report, despite the fact that we are confident in our hypothesis. This does not mean that the hypothesis is bulletproof (hypotheses are never bulletproof), and we see two additional pieces of evidence that would help to further strengthen our argument. First, we would like to examine the type specimen of *C. reyesi*. Unfortunately, this specimen, originally deposited at the American Museum of Natural History, cannot be located at this time (AMNH, personal communication). However, we note that illustrations of this specimen by Gertsch (1992), and examination of newly collected specimens from the type locality (Airman’s cave), are consistent with the synonymy arguments that we have

made. Ultimately, if *C. cueva*, *C. bandida*, and *C. reyesi* are synonymized as we propose, all populations within this complex will be referred to as *C. bandida*, as this name has page priority (Gertsch 1992).

We would also like to have DNA sequence data for specimens from Bandit's Cave, the type locality of *C. bandida*. Unfortunately, access to this cave was not granted for our study. Again, the morphology of the type specimen from Bandit's cave is consistent with our hypothesis, and we do in fact have DNA data from caves close to Bandit's cave. Again, all available data are consistent with the synonymy arguments that we have made.

Finally, it would be useful to collect nuclear DNA data from multiple populations in the *C. cueva* species complex, plus relevant outgroups. Our prediction is that these data would reveal a genetic clade corresponding to the complex, but with less internal genetic structuring (nuclear data evolves generally more slowly than mtDNA data).

Acknowledgements:

This project benefited from the help of numerous persons. We would like to first thank Nadine Dup  r   for her outstanding capacity to generate so many wonderful illustrations and to Cor Vink for his enthusiasm and collaboration in the lab. Fieldwork would not have been possible without the help of Mark Sanders (City of Austin), Cyndee Baker (U.S. Fish and Wildlife) and Kemble White (SWCA). Several other also helped to provide cave access, company in the field, and a friendly hand for collection: Gerry Fant, Rose Farmer (Travis County), Nico Hauwert (City of Austin), Julie Jenkins (TCMA), Jean Krejca, Cal Noonan (TXDOT), Kathleen O'Connor (Travis County), Robert Pine (U.S. Fish and Wildlife), Kevin Thuesen (City of Austin), Brad and Spike Robinson, Bill Russel (TCMA), Alisa Shull (U.S. Fish and Wildlife), Chris Thibodaux, Mike Walsh (TCC), and Jenny Wilson (U.S. Fish and Wildlife). Insights into Texas caving benefited from discussions with Mike Warton, James Reddell, Jean Krejca and James Cokendolpher. Special thanks go to James Reddell for sharing his thoughts and knowledge about cave life. Thanks to Norman Platnick and Lou Sorkin (American Museum of Natural History) for the loan of the type specimens of *C. cueva* and *C. bandida*. Thanks to Jose Macias and Steven Thomas for their interest and help in the lab. Finally, thanks also to the folks from the Austin Motel.

References:

- Gertsch WJ. 1992. Distribution patterns and speciation in North American cave spiders with a list of the troglobites and revision of the cicurinas of the subgenus *Cicurella*. Texas Memorial Museum Speleological Monographs, 3. Studies on the endogean fauna of North America, II, 75-122.
- Paquin, P. and M.C. Hedin. 2004. The power and perils of “molecular taxonomy”: a case study of eyeless and endangered *Cicurina* (Araneae: Dictynidae) from Texas caves. *Molecular Ecology* 13, 3239-3255.

Table 1 - Summary of genetic distances within and between species. All data are from Paquin & Hedin (2004), except for the *C. cueva* complex data.

	Species	N	Within	Between
S U R F A C E	<i>C. arcuata</i>	2	0.1	10.06
	<i>C. bryantae</i>	2	0.91	8.84
	<i>C. breviararia</i>	2	3.09	8.84
	<i>C. pacifica</i>	1	-	10.06
	<i>C. pallida</i>	2	0	9.2
	<i>C. pampa</i>	1	-	12.03
	<i>C. placida</i>	2	0	9.15
	<i>C. varians</i>	22	2.54	10.98
C A V E	<i>C. brunsi</i>	1	-	8.33
	<i>C. bullis</i>	7	1.63	6.32
	<i>C. caliga/hoodensis</i>	14	0.41	1.73
	<i>C. coryelli</i>	1	-	3.55
	<i>C. buwata</i>	2	0	3.15
	<i>C. ezelli</i>	2	0	3.15
	<i>C. loftini</i>	2	0	6.32
	<i>C. madla/vespera</i>	22	3.96	10.06
	<i>C. mixmaster</i>	1	-	1.73
	<i>C. puentecilla/platypus</i>	7	2.35	4.67
	<i>C. reddelli</i>	1	-	8.39
	<i>C. reyesi</i>	2	0	8.47
	<i>C. troglobia</i>	1	-	7.01
	<i>C. vibora</i>	4	1.42	7.03
	<i>Cicurina sp 1</i>	1	-	4.67
	<i>C. cueva complex</i>	51	3.96	6.17

Notes: Within species distances reported as the *maximum* pairwise sequence divergence found within a given species, estimated as p distances (the observed proportion of sites that differ between sequences) X 100. Between species divergences are *minimum p* distances (X 100) from one species to the nearest (in genetic distance) phylogenetic relative. N represents the number of sequences sampled for each species.

Figure Legends:

Figure 1 – mtDNA phylogeny of eyeless *Cicurina*, inferred using the NJ algorithm. Specimens outside the primary group of interest (the *C. cueva* complex) include “outgroup” taxa from caves just south of the primary region of interest (see **Fig. 3**), plus more distantly related species. The individuals included from these more distantly-related species are from type localities, as follows: Beck’s Sewer Cave (*C. buwata*), Tooth Cave (*C. travisiae*), Brown’s cave (*C. browni*), Caracol Creek Coon Cave (*C. loftini*), Temples of Thor (*C. vibora*), and Lakeline Cave (undescribed species).

Figure 2 – Phylogeny of mtDNA sequences for members of the *C. cueva* complex plus close outgroups, inferred using unpartitioned Bayesian methods. Posterior probabilities (measures of support ranging from 0 – 1.0) are given for each of three replicate analyses. Partitioned Bayesian and maximum likelihood analyses give similar results. Colored bars correspond to phylogenetic sequence subclades that are recovered in all phylogenetic analyses conducted. Red dots indicate caves where sequenced specimens fall into more than one phylogenetic subclade. Blue dots indicate specimens collected from Ireland’s Cave – these specimens fall into three different subclades.

Figure 3 – Map showing distribution of sampled populations of the *C. cueva* complex, plus close outgroups to the south. Some cave locations are intentionally not shown (Barker Ranch Cave, Flint Ridge Cave, County Line Bat Cave and Hoskin’s Hole). Map colors correspond to the subclades found in phylogenetic analyses (see Fig 2) – individuals from Barker Ranch Cave, Flint Ridge Cave, and County Line Bat Cave fall into the purple subclade. Red dots indicate caves with individuals that carry haplotypes from more than one genetic subclade. The blue dot indicates Ireland’s Cave. The hypothesized distribution of the *C. cueva* complex is outlined by a dashed line.

Figure 4 – This figure illustrates morphological variation observed *within and between populations within a single species*, versus the variation observed between more distantly-related species. These drawing are of female spermathecae, a sclerotized cuticular structure that is found on the ventral surface of adult female Entelegyne spiders, just underneath the primary exoskeleton. The spermathecae includes connecting canals,

plus a primary bulb (the sperm storage structure itself). This figure illustrates that the shape of the connecting canal is variable (red arrows), even within specimens from the same cave (see *C. travisiae* complex). This contrasts with the shape of the spermathecal bulb (blue arrows), which is more conservative within species, but varies obviously across species. Plates on the left are in ventral view, plates on the right are in dorsal view (i.e., the structure has been dissected from the spider, and is viewed from the top, as if from inside the spider).

Figure 5 – Original illustrations of type specimens of *C. bandida*, *C. cueva*, and *C. reyesi* (by Gertsch 1992). These are compared directly to re-illustrations of the type specimens of *C. bandida* and *C. cueva*, plus an illustration of a specimen from the type locality of *C. reyesi* (= Airman’s Cave) collected in this study. We did not re-illustrate the type specimen of *C. reyesi* because it could not be located at the AMNH.

Figure 6 – This figure summarizes our efforts to place female specimens into morphological groups, based on a **qualitative** consideration of the curvature and shape of the connecting canal (see red lines), observed in **ventral view**. We were as generous as possible in this clustering (i.e., allowing for some variation within groups). We defined five potential morphological groups based on the variation observed. The phylogenetic and geographic position of these specimens (as indicated by the colored bars, following **Figs. 2 & 3**) reveals no obvious relationship between morphology, geographic origin, and position on an mtDNA phylogeny. Each group that includes more than one specimen has no obvious geographic or phylogenetic cohesion.

Figure 7 – This figure summarizes our efforts to place female specimens into morphological groups, based on a qualitative consideration of the distance between the top of the spermathecal bulb and the upper loop of the connecting canal, observed in **dorsal view** (see blue arrows). Four groups were determined: 1 = spermathecal bulb lying above upper loop, 2 = upper loop lying just above bulb, 3 = small gap between bulb and upper loop, 4 = larger gap between bulb and upper loop. The geographic and phylogenetic position of these specimens (as indicated by the colored bars, following Figs. 2 & 3) reveals no obvious relationship between morphology, geographic origin, and position on an mtDNA phylogeny. Each group that includes more than one specimen has no obvious geographic or phylogenetic cohesion.

Figure 8 – Adult female spermathecal morphology (in **ventral view**) mapped onto the mtDNA tree (see Fig. 1). This figure illustrates the range of variation observed within the *C. cueva* complex, plus the lack of any obvious association between morphology and phylogeny.

Figure 9 – Adult female spermathecal morphology (in **dorsal view**) mapped onto the mtDNA tree (see Fig. 1). This figure illustrates the range of variation observed within the *C. cueva* complex, plus the lack of any obvious association between morphology and phylogeny.

Figure 1

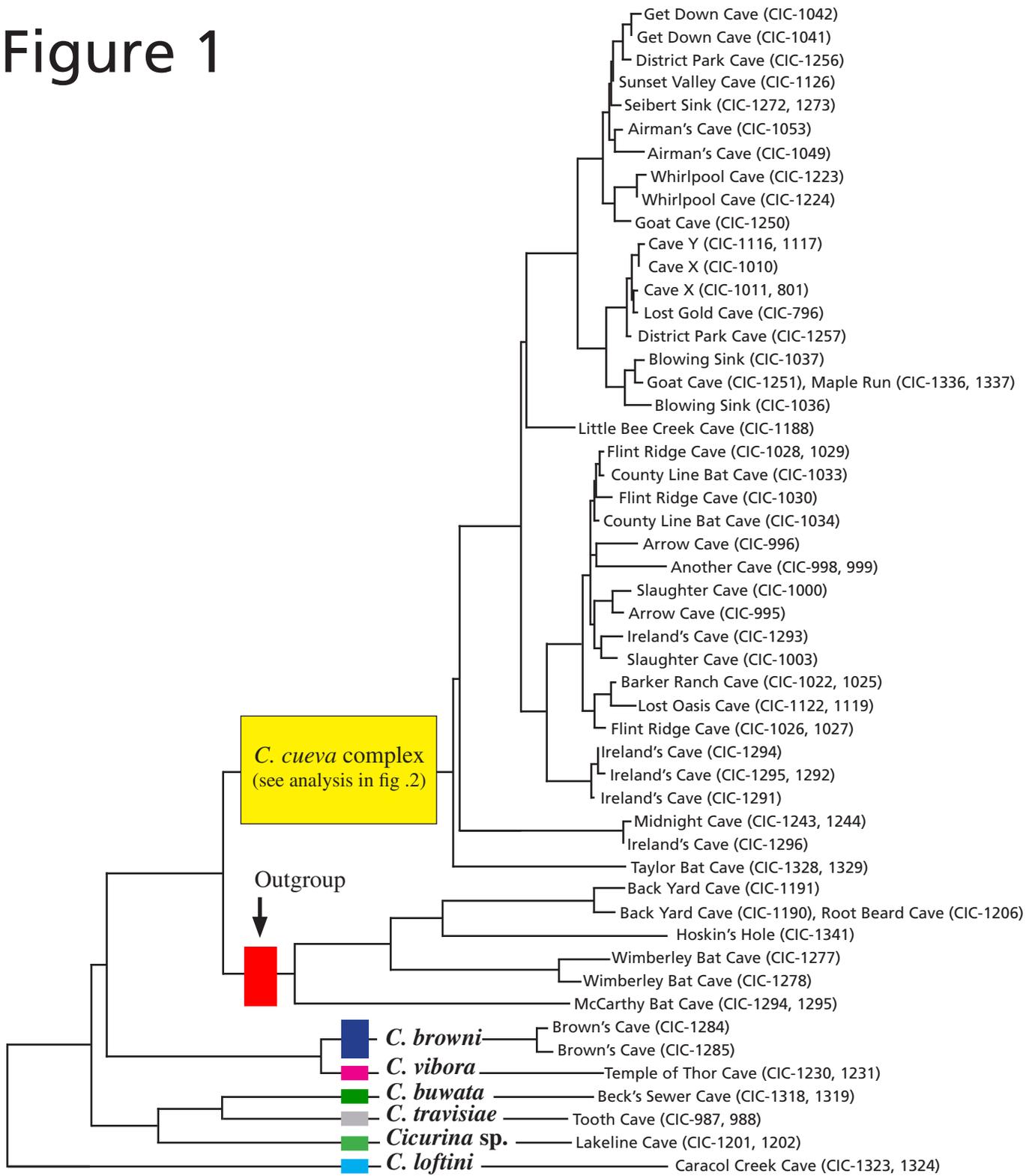


Figure 2

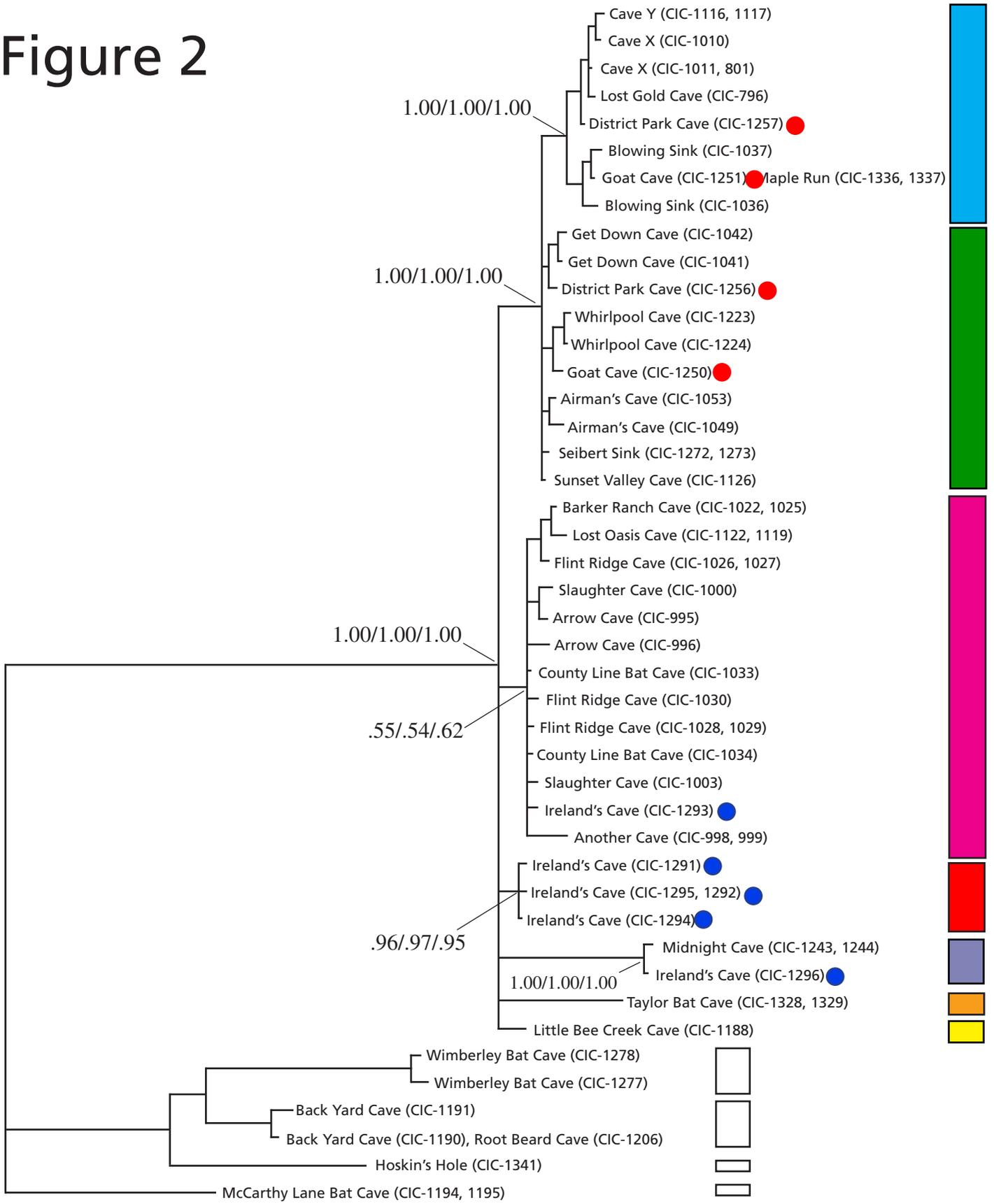
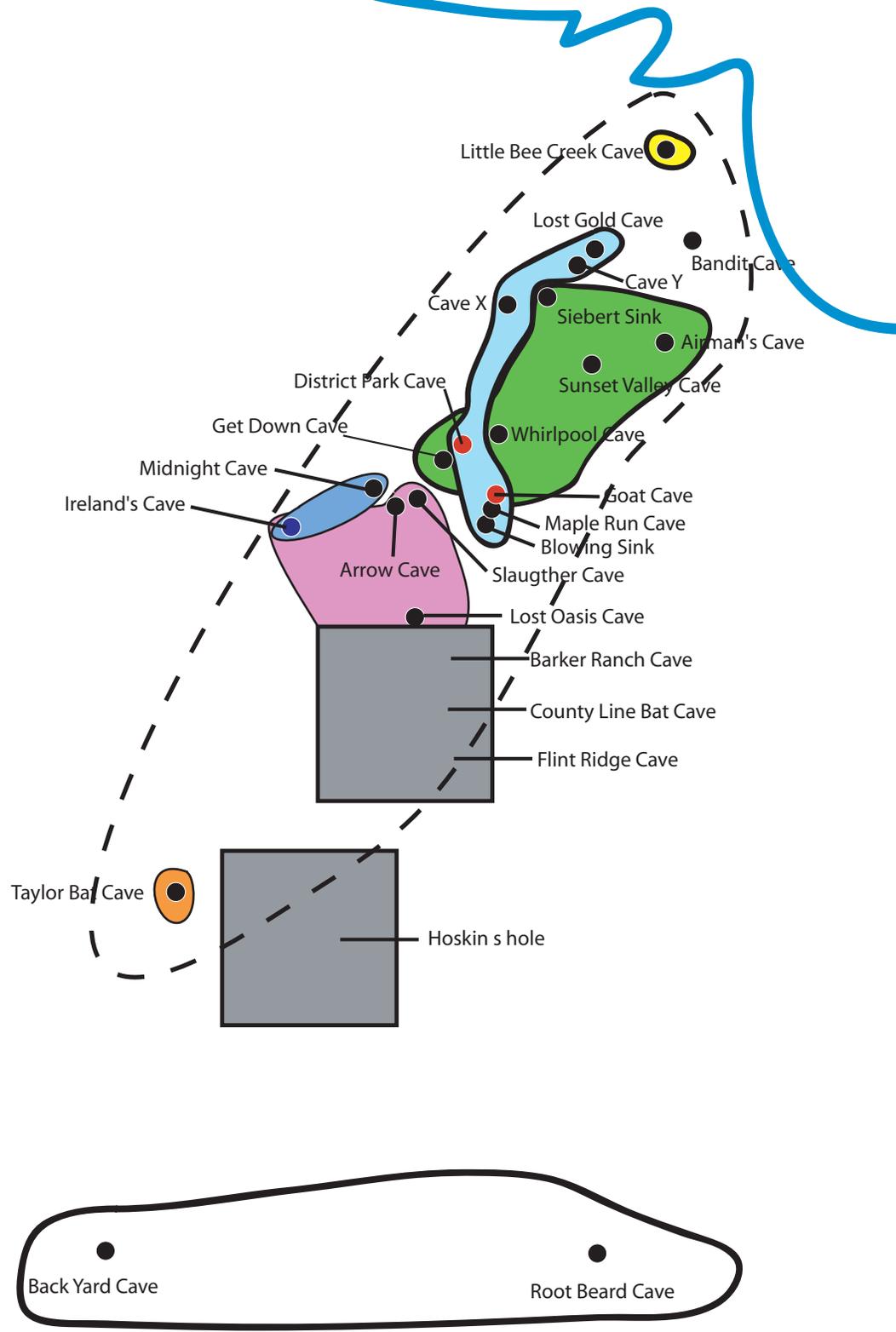


Figure 3



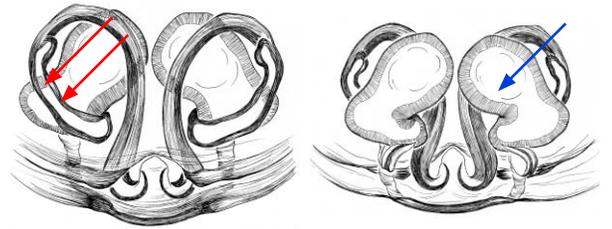
Wimberley Bat Cave



McCarthy Line Bat Cave

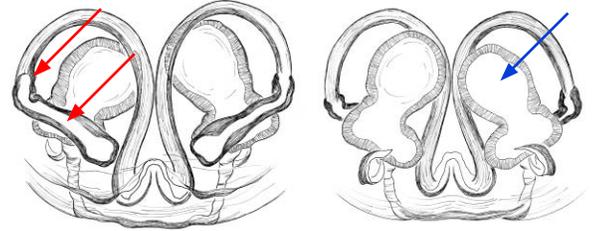
Figure 4

Cave X (CIC-1010)

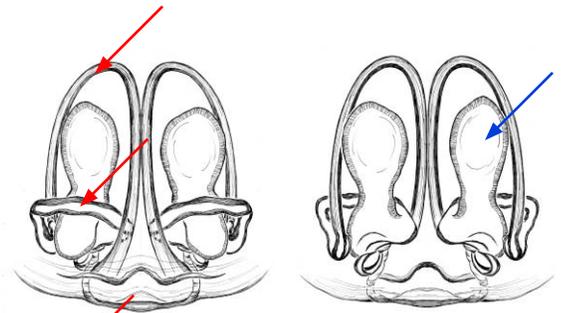


C. cueva complex

Lost Oasis Cave (CIC-1119)

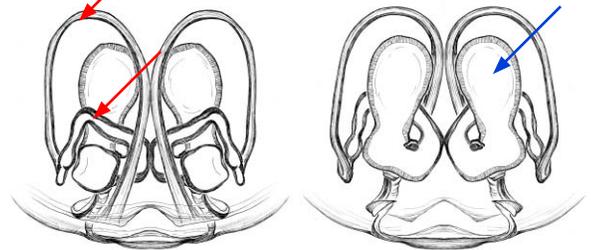


Whitewater Cave (CIC-1013)

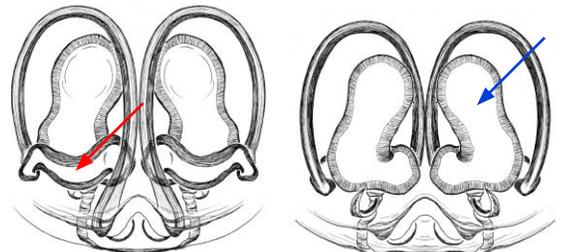


C. buwata complex

Nelson Ranch Cave (CIC-1018)



McDonald's Cave (CIC-1099)



C. travisiae complex

McDonald's Cave (CIC-1102)

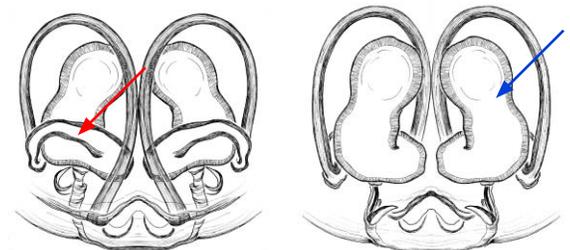


Figure 5

Cicurina cueva, **type specimen** re-illustrated

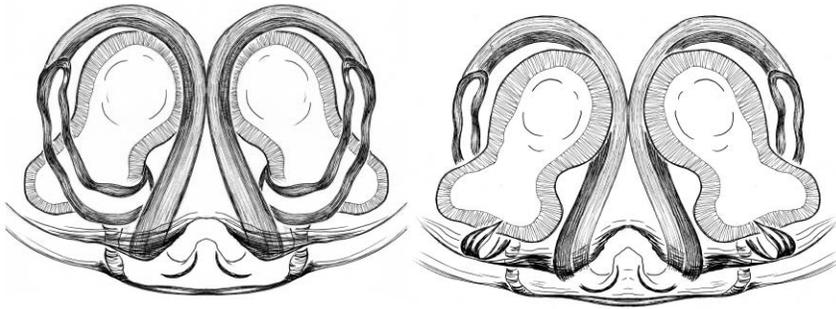
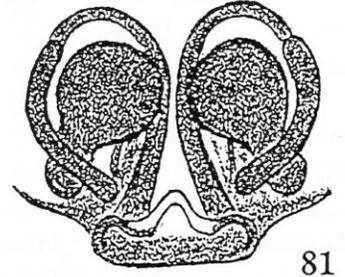


Illustration of the same specimen by Gertsch (1992)



Cicurina bandida, **type specimen** re-illustrated

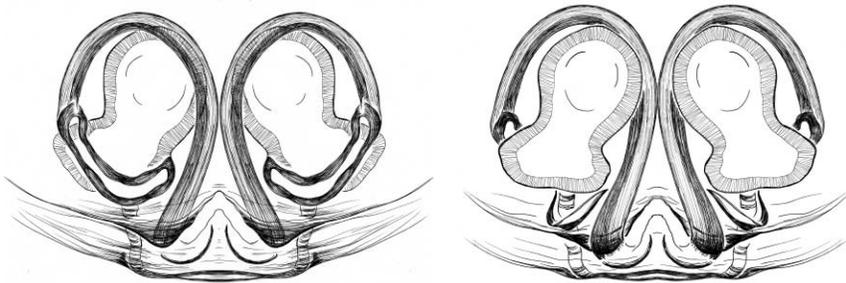
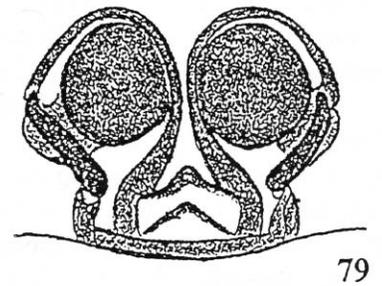


Illustration of the same specimen by Gertsch (1992)



Cicurina specimen collected from Airman's Cave, the **type locality** of *C. reyesi*

Illustration of the type specimen by Gertsch (1992), the only other known adult specimen from Airman's Cave

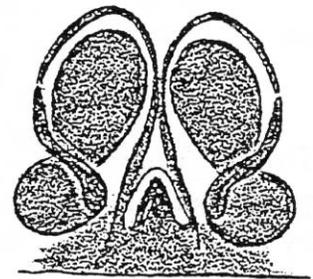
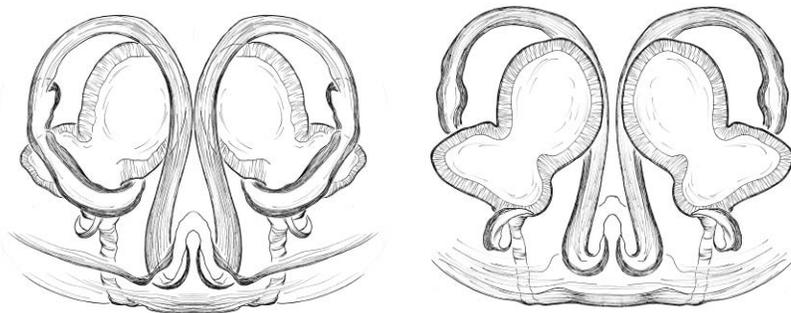
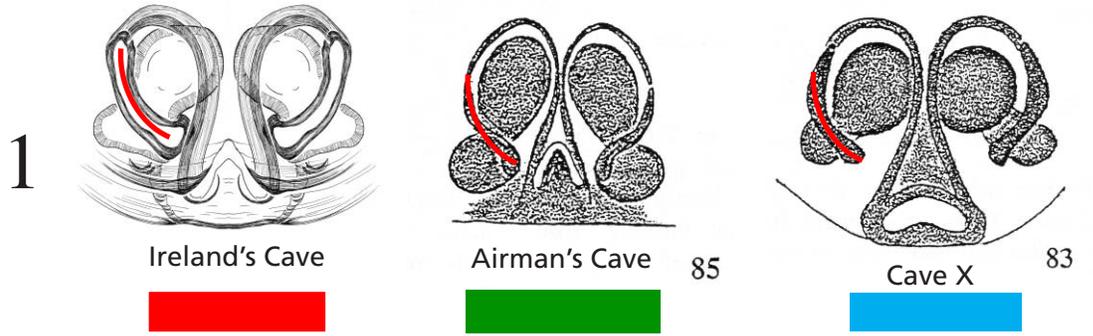
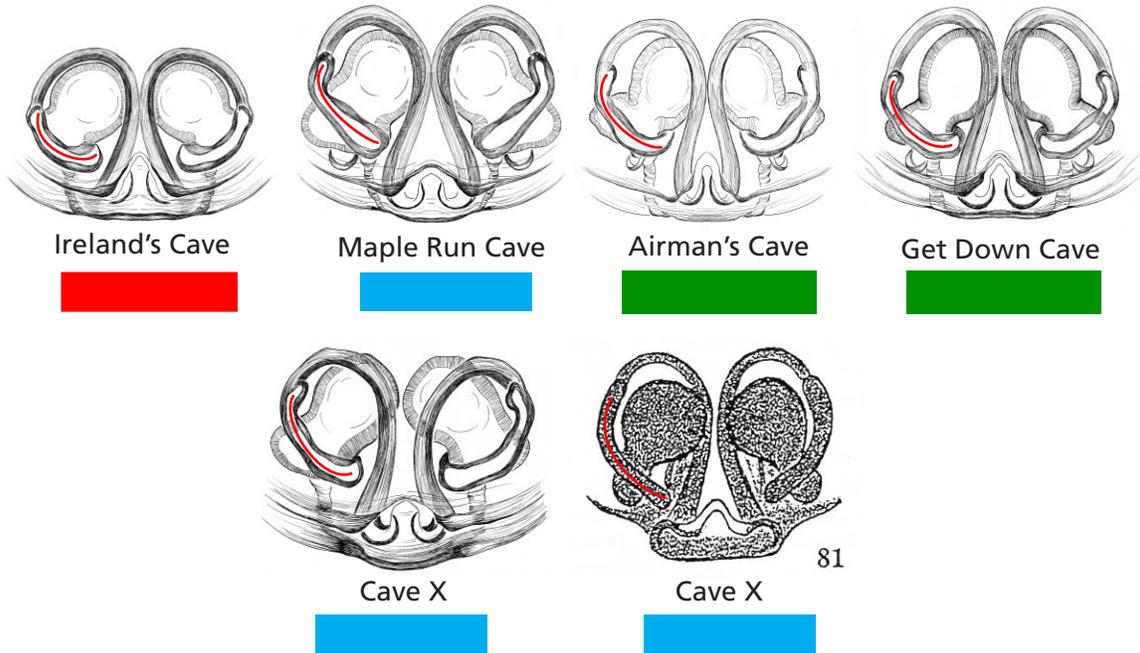


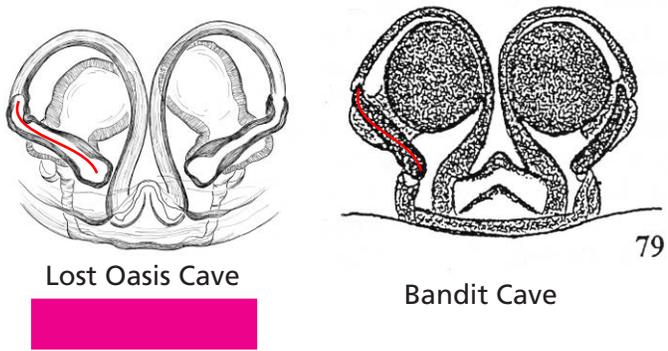
Figure 6



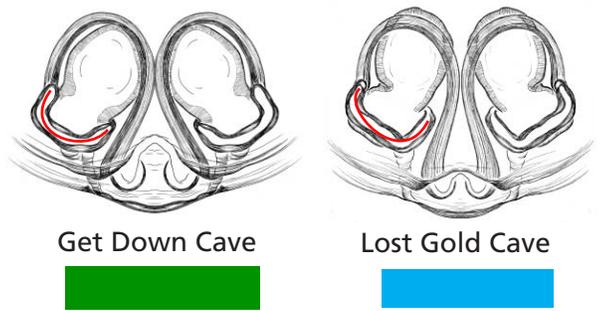
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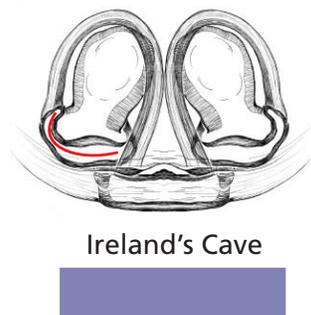
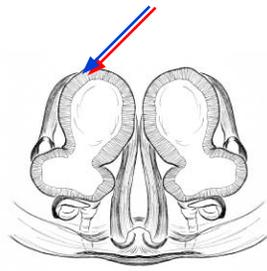


Figure 7

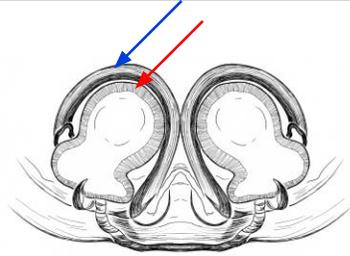
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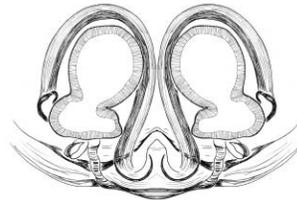
Lost Gold Cave



2



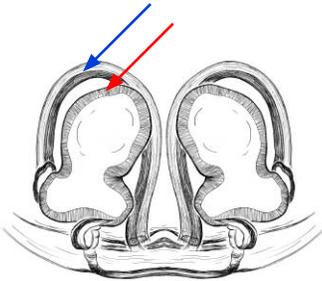
Ireland's Cave



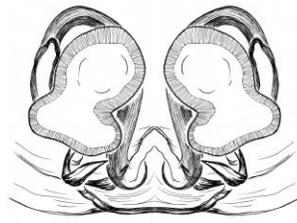
Get Down Cave



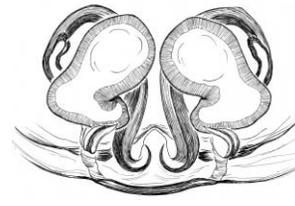
3



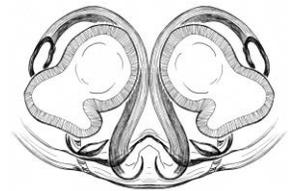
Ireland's Cave



Ireland's Cave



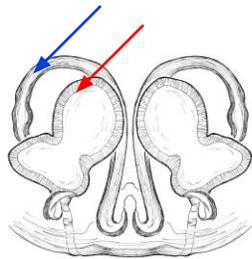
Cave X



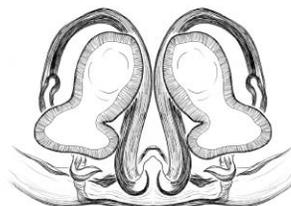
Maple Run Cave



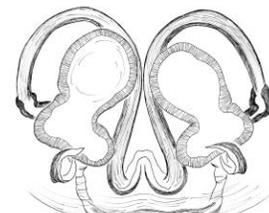
4



Airman's Cave



Get Down Cave



Lost Oasis Cave



Figure 8

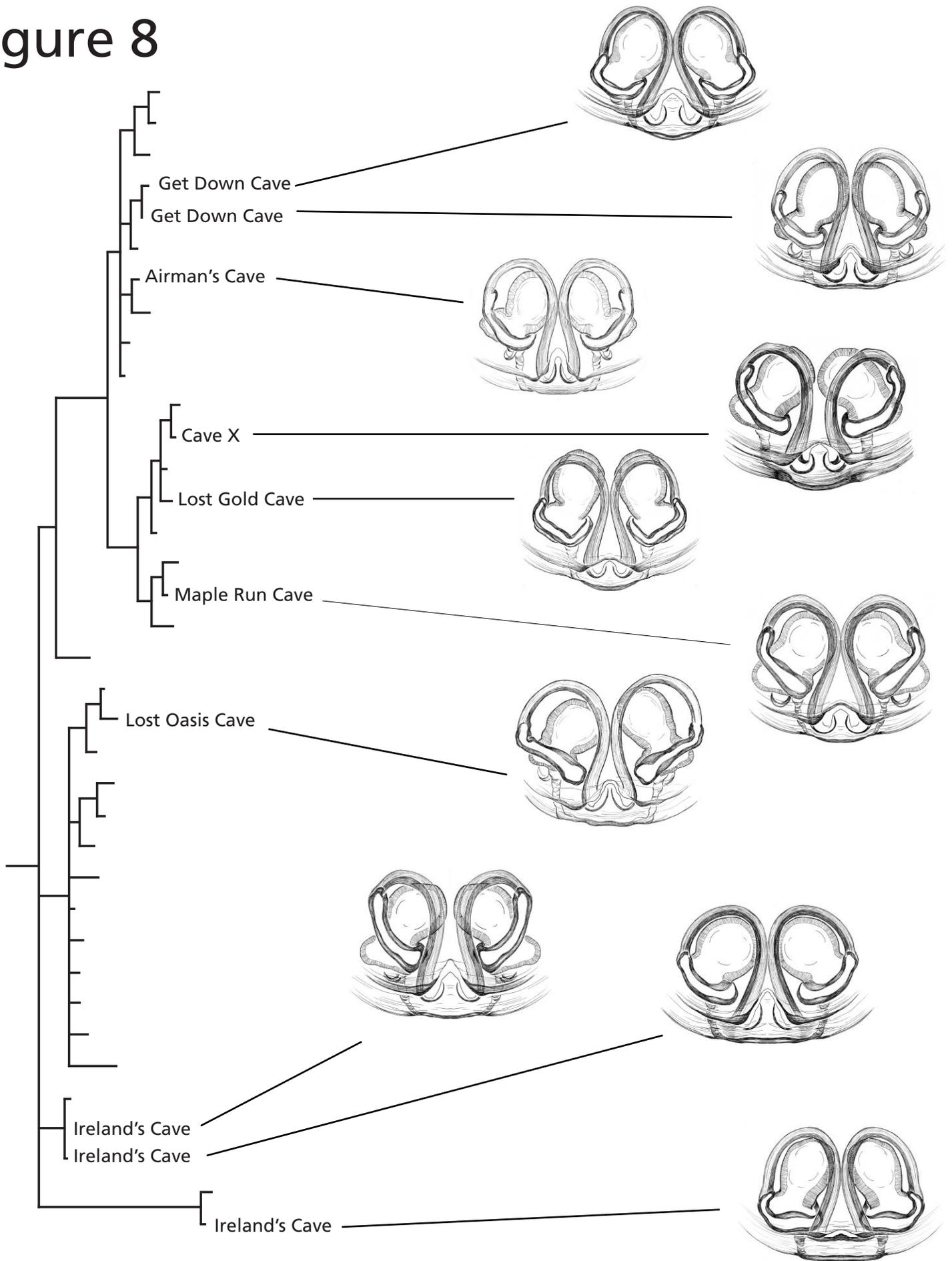


Figure 9

